# A novel role for Rab5–GDI in ligand sequestration into clathrin-coated pits

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**Background**: Clathrin-coated pits are formed at the plasma membrane by the assembly of the coat components, namely clathrin and adaptors from the cytosol. Little is known about the regulation and mechanism of this assembly process.

**Results**: We have used an *in vitro* assay for clathrin-coated pit assembly to identify a novel component required for the invagination of newly formed coated pits. We have purified this cytosolic component and shown it to be a complex of Rab5 and GDI (guanine-nucleotide dissociation inhibitor), that was previously demonstrated to be involved in downstream processing of endocytic vesicles. Using a combination of quantitative electron microscopy and *in vitro* endocytosis assays, we have demonstrated that although coat proteins and ATP are sufficient to increase the number of new coated pits at the cell surface in permeabilised cells, the Rab5–GDI complex is required for ligand sequestration into clathrin-coated pits.

**Conclusions**: We have identified Rab5 as a critical cytosolic component required for clathrin-coated pit function. Given the well-established role of Rab5 in the fusion of endocytic vesicles with endosomes, our results suggest that recruitment of essential components of the targeting and fusion machinery is coupled to the formation of functional transport vesicles.

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## Background

The cellular uptake of many biologically important macromolecules is mediated by clathrin-coated pits and coated vesicles. Coated pits assemble at the plasma membrane following recruitment of coat proteins from the cytosol [1–3]. The coat is composed of clathrin, which acts as a structural scaffold [4], and a complex of adaptor proteins, which form an inner layer linking transmembrane receptors to the clathrin lattice [5]. The adaptor complex AP2 is found specifically in plasma membrane coated pits and vesicles, whereas AP1 is localised to clathrin-coated vesicles which bud from the trans-Golgi network [6]. Studies of cells that have been freeze-fractured and deep-etched reveal that clathrin initially assembles onto the membrane as a planar lattice composed of hexagons and, as the lattice becomes increasingly invaginated, there is a conversion of hexagons to pentagons [7,8]. Little is known about the molecular mechanisms that drive clathrin-coated pit assembly and coated vesicle formation [9]. However, the recent development of cell-free systems that measure distinct and overlapping stages in coated pit and vesicle formation [10–12] has created an opportunity to identify the essential components that regulate these processes. Previous studies using this approach have demonstrated that both the formation of new coated pits and the budding process which forms coated vesicles require cytosolic components and ATP [10,11]. Clathrin-coated pit assembly is stimulated by purified AP2 adaptor complexes, but only in the presence of clathrin and cytosol [12], indicating that, in addition to the known structural components of the coat, other cytosolic components are required on the pathway to vesicle formation. These might include factors required downstream of vesicle formation, for example, for correct vesicle targeting and/or fusion.

The targeting of transport vesicles such as clathrin-coated vesicles to their acceptor compartment is tightly regulated. One class of specific proteins involved in the maintenance of targeting fidelity is the Rab family, comprising at least 30 small GTP-binding proteins that are known to be involved in the regulation of membrane trafficking in the endocytic and exocytic pathways [13]. Members of the Rab family show distinct subcellular localisations [14], and various transport processes have been shown to be dependent on functional Rab proteins [15], but their precise mechanism of action has yet to be fully elucidated. Studies in yeast have indicated that Rab proteins interact with and activate SNARE proteins (soluble N-ethylmaleimide-sensitive fusion protein attachment protein (SNAP) receptors) [16,17]. SNARE proteins are integral membrane proteins which confer vesicle targeting specificity, and comprise two families: v-SNAREs are thought to direct transport vesicles

to their correct target membrane where the v-SNARE interacts with a cognate t-SNARE. Although implicated in the assembly of SNARE complexes, Rab proteins have not, however, been detected in these complexes and a role for Rabs as catalysts in v-SNARE and t-SNARE interactions has been proposed [18–20]. It is also postulated that Rab proteins have a proof-reading function which maintains the fidelity of transport by ensuring that there is correct pairing between v-SNARE and t-SNARE partners.

Rab5 has been localised to early endosomes and the plasma membrane. Early studies using *in vitro* assays that reconstitute early endosome fusion [21] indicated that Rab5 is essential for homotypic fusion [22]. Overexpression of wild-type Rab5 enhanced the rate of transferrin internalisation, and overexpression of Rab5 mutants deficient in GTP binding inhibited transferrin uptake [23]. These findings suggested that Rab5 activity is rate-limiting in the early endocytic pathway. Overexpression of the wild-type protein caused an accumulation of large endosomal structures, which differed from the small vesicular structures seen in cells overexpressing the mutant protein. Rab5 was also shown to be a component of clathrin-coated vesicles [23]. Taken together, these results indicated a role for Rab5 in fusion between clathrin-coated vesicles and early endosomes.

Rab proteins are present in both membrane-bound and soluble pools. In the cytosol, these proteins are complexed to Rab guanine-nucleotide dissociation inhibitor (GDI) [24]. GDI binds to Rabs that are in the GDP-bound, inactive conformation and targets these proteins to their site of action. GDI then dissociates from the membrane and a guanine-nucleotide exchange factor mediates GTP/GDP exchange on the membrane-bound Rab protein [25,26]. This chaperone activity of GDI is important in directing Rab proteins to the correct target membrane. Rab proteins that are complexed to delipidated bovine serum albumin can be delivered to membranes, but the specificity of delivery is lost [27]. It is as yet unclear how GDI confers specificity to this delivery process.

In this study, we have demonstrated that coat proteins, in the presence of an energy source, are sufficient for the formation of new coated pits in permeabilised A431 cells. A cytosolic component(s) is, however, essential for these clathrin-coated pits to sequester transferrin efficiently. We have purified this component and shown it to consist of a complex of Rab5 and GDI (Rab5–GDI). These results indicate a novel role for the Rab5–GDI complex at the very earliest stages of the endocytic pathway. Furthermore, we show that Rab proteins are required at all stages on the pathway to coated vesicle formation. Thus, there appears to be a direct link between the processes of transport vesicle formation and the recruitment of components required for subsequent fusion reactions.

## Results

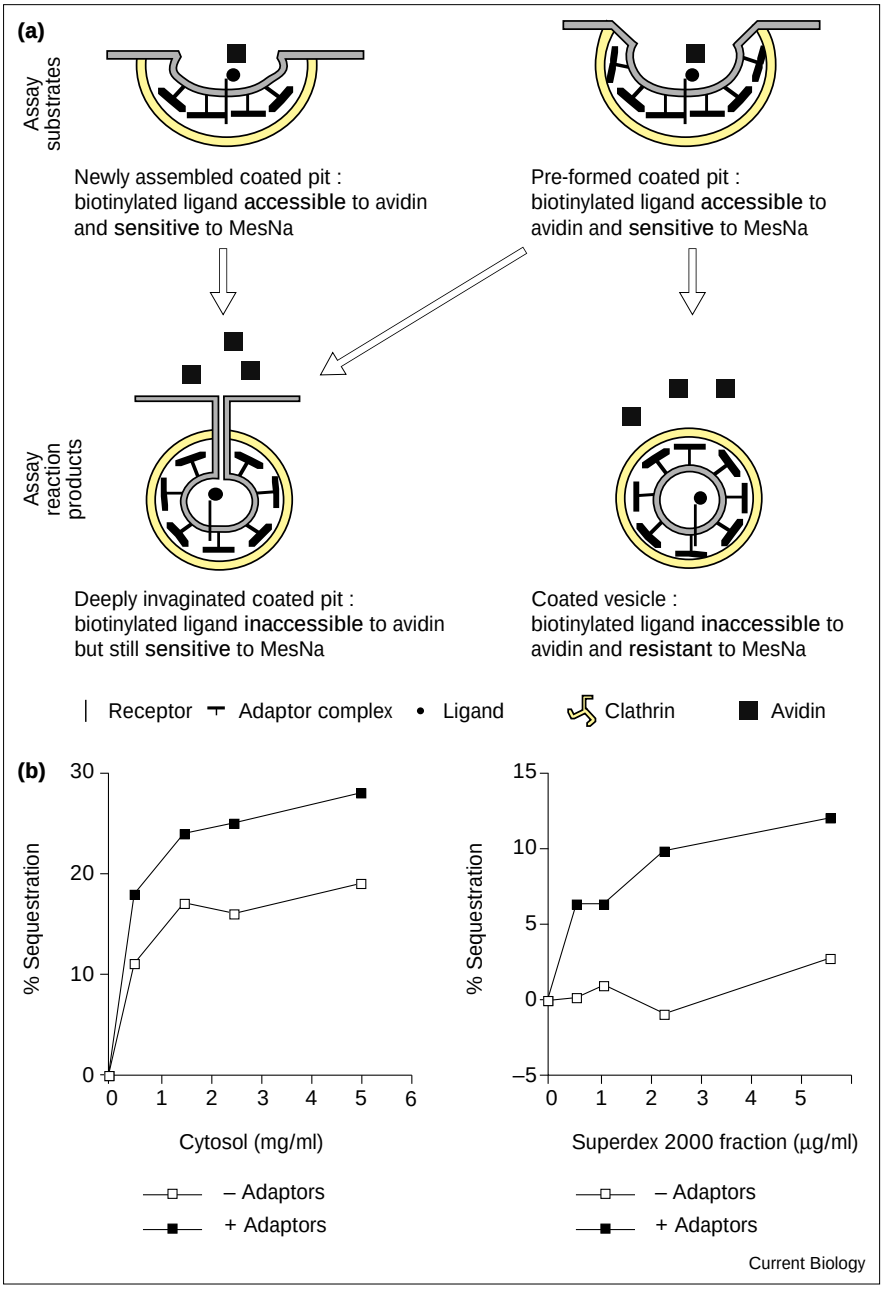
### Rab5–GDI is the cytosolic component that supports adaptor-dependent coated pit assembly

Stage-specific assays in permeabilised A431 cells have been developed that measure different and overlapping events on the pathway to clathrin-coated vesicle formation. A derivative of transferrin termed BSST, which is biotinylated via a cleavable disulphide linkage, is used as a reporter molecule. Both the sequestration of BSST into deeply invaginated coated pits and its internalisation into coated vesicles are measured by a loss of accessibility of BSST to exogenously added avidin. The internalisation of BSST into clathrin-coated vesicles also renders it resistant to the small membrane impermeant reducing agent MesNa [11]. From previous studies, we know that inaccessibility to avidin measures new coated pit formation, the growth and invagination of existing pits, and the scission step that gives rise to coated vesicles; in contrast, the MesNa-resistance assay measures only the late stages of coated vesicle budding [10,11]. Figure 1a illustrates schematically the 'substrates' and reaction products measured by the different *in vitro* assays.

Both the assembly and the budding of coated pits are dependent on cytosolic factors. Adaptors are limiting in our cytosolic preparations, so the early stages of coated pit assembly may be stimulated by purified adaptor proteins in a cytosol-dependent and clathrin-dependent manner [12]. In order to elucidate further the mechanism of coat assembly, we aimed to purify the cytosolic component(s) essential for this process. Bovine brain cytosol was fractionated, and the fractions were assayed for their ability to support adaptor-dependent transferrin sequestration using the avidin-inaccessibility assay. Figure 1b illustrates a typical titration curve for cytosol in the presence and absence of adaptors. In order to quantify the activity present in cytosol and cytosolic fractions, we assayed the extent of transferrin sequestration in the presence and absence of adaptors over a range of concentrations of cytosol and cytosolic fractions. Subtraction of the curve observed in the absence of adaptors from that seen in the presence of adaptors gave a measure of adaptor-dependent transferrin sequestration only. We arbitrarily defined a unit of activity as that amount of protein which gave half-maximal adaptor-dependent transferrin sequestration. In the absence of cytosol, the extent of sequestration in the presence of adaptors and ATP was not significantly greater than that observed in the presence of ATP alone. Using this assay system, we have achieved a 2000-fold purification of a cytosolic activity essential for adaptor-dependent ligand sequestration to a recovery of approximately 2% (Table 1).

Unfractionated bovine brain cytosol (Figure 1b) supported considerable basal transferrin sequestration in the absence

**Figure 1**




*In vitro* endocytosis assays. (a) Intermediates measured by *in vitro* assays for endocytosis. The 'substrates' can be either newly assembled clathrin-coated pits or pits already present when the cells are permeabilised. Both of these populations contain transferrin receptors and can therefore bind biotinylated ligand, which will be accessible to both avidin and MesNa. Invagination of both of these populations of pits results in the sequestration of ligand into deeply invaginated structures such that the ligand is no longer accessible to avidin. Scission of deeply invaginated coated pits to form coated vesicles results in the biotinylated ligand becoming inaccessible to avidin and resistant to MesNa. It appears that only a 'primed' population of pits already present in permeabilised cells is capable of undergoing scission. (b) Assay for adaptor-dependent transferrin sequestration. Bovine brain cytosol or the Superdex 200 pool were titrated in the presence of an ATP-regenerating system and the presence or absence of adaptors, and the amount of biotinylated transferrin sequestered was measured using the avidin inaccessibility assay as described in Materials and methods. Background sequestration observed in the presence of an ATP-regenerating system and in the absence of cytosol (~20% of total) have been subtracted in each case. The extent of sequestration seen in the presence of adaptors and the absence of cytosol or cytosolic fractions did not exceed the amount of sequestration seen in the presence of ATP alone.

of added adaptors. This basal activity is lost from more purified fractions (Figure 1b). Thus, both the purified cytosolic activity and the adaptor complexes are required for ligand sequestration. Although the overall efficiency of sequestration drops in the presence of purified fractions and adaptors compared with that in the presence of cytosol and adaptors, a comparison of the curves obtained in the absence and presence of adaptors in Figure 1b shows that the adaptor-dependent stimulation remains the same at 10–15% sequestration. This finding argues that the purified activity fulfils all of the cytosolic requirements for adaptor-dependent transferrin sequestration in the permeabilised A431 cell system. In addition, at each step of the purification procedure we added inactive fractions to the active fraction in the assay and in no case did we observe an enhancement of activity.

The final step in the purification procedure was chromatography through a Superdex 200 gel filtration column. Analysis of the eluted fractions by SDS–PAGE revealed that the activity co-eluted with two bands of molecular weight 58 kDa and 27 kDa (Figure 2a). This preparation

**Table 1**

**Purification of cytosolic activity for adaptor-dependent sequestration of transferrin into invaginated coated pits.**

| Step | Units | Specific Activity (Units/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|
| Cytosol | 205,000 | 50 | 1 | 100 |
| Reactive red | 54,998 | 1,742 | 35 | 27 |
| MonoQ | 36,666 | 5,555 | 111 | 18 |
| Phosphocellulose | 7,920 | 10,000 | 200 | 3.9 |
| Superdex 200 | 4,455 | 100,000 | 2,000 | 2.2 |

The table shows a typical purification table from 1 kg of bovine brain. Units are defined in Results.

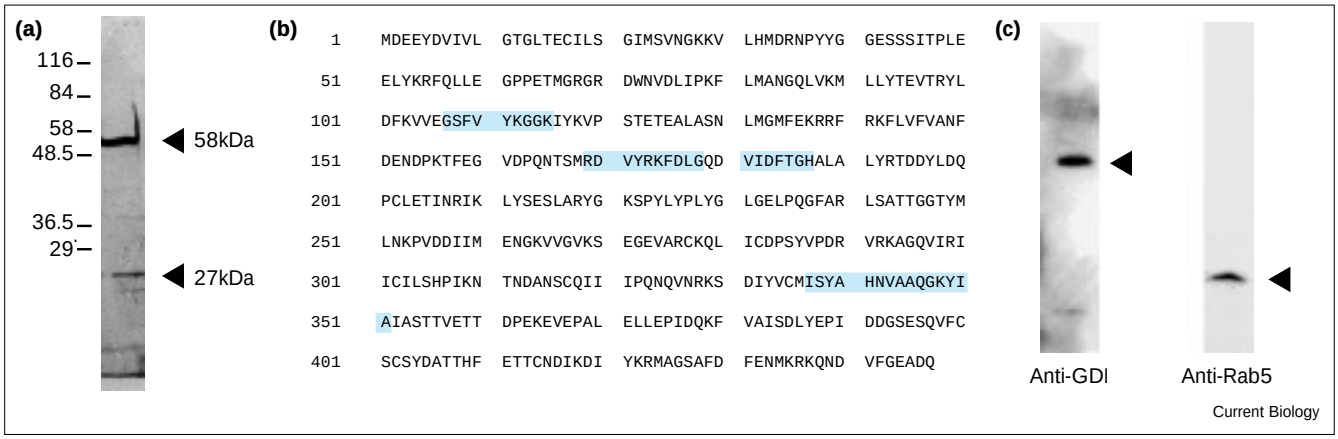
was subjected to in-solution digestion with cyanogen bromide and, after western blotting, amino-terminal sequence from four fragments was obtained. Comparison of these sequences to sequences in the SwissProt database revealed 100% identity to RabGDIα (Figure 2b). To confirm the identity of the 58 kDa band, antiserum was raised against peptides corresponding to distinct regions of GDI (see Materials and methods). This antiserum cross-reacted with the 58 kDa band on western blots (Figure 2c).

One of the well-documented functions of GDI is to chaperone Rab proteins, which are required for the fidelity of transport, to the correct target membrane. The presence of a 27 kDa band in the final active fraction suggested that this protein might be a member of the Rab family. Given the well-established role of Rab5 in the early endocytic pathway and its localisation to the plasma membrane and early endosomes [28], it seemed a likely candidate for the 27 kDa protein. This protein was confirmed to be Rab5 by western blotting using a monoclonal antibody to Rab5 (Figure 2c).

To confirm that GDI was indeed a component of the activity required for adaptor-dependent transferrin sequestration, we used affinity-purified anti-peptide antibodies against GDI (see Materials and methods) to specifically deplete GDI from cytosol (Figure 3a). Treatment of cytosol with irrelevant sheep serum coupled to protein-G–Sepharose reduced adaptor-dependent transferrin sequestration to 80% of control (untreated cytosol). A similar reduction in activity was observed in the presence of protein-G–Sepharose alone (data not shown). Depletion of GDI from cytosol using anti-peptide 1 antibodies abolished adaptor-dependent transferrin sequestration completely, and depletion with anti-peptide 2 antibodies substantially inhibited (> 60%) adaptor-dependent sequestration (Figure 3b). For both antibodies, the extent of inhibition observed in the *in vitro* endocytosis assay correlated well with the degree of depletion of GDI from the fraction (Figure 3a).

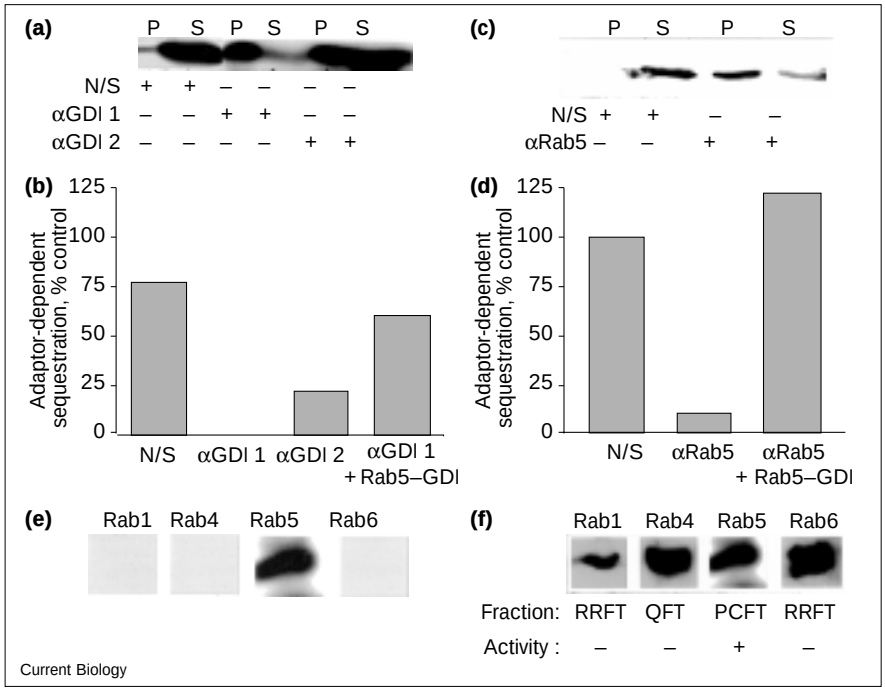
The 27 kDa band in our most purified fraction which cross-reacted with anti-Rab5 antibodies was the major polypeptide in the molecular weight range 20–30 kDa. To confirm that the activity was specific for Rab5 rather than other Rab proteins, we immunodepleted Rab5 from

**Figure 2**




The cytosolic activity required for adaptor-dependent transferrin sequestration is a complex of Rab5–GDI. **(a)** Analysis by 10% SDS–PAGE of the purified active fraction after gel filtration through Superdex 200. The gel was silver-stained and the molecular weights of standards (in kDa) are as indicated. The positions of the 58 kDa and 27 kDa bands are indicated by arrows. **(b)** Sequence of bovine brain RabGDIα. The peptides that were sequenced from our preparation are indicated by the boxed regions. Two of the peptides sequenced (residues 106–115 and residues 171–177) showed an atypical amino-terminal sequence for cyanogen-bromide cleavage and we assume that this sequence arose as a result of acid cleavage of the peptides during digestion. **(c)** Western blot of the active fraction shown in (a). Arrows indicate a band at 58 kDa which cross-reacts with anti-GDI antibodies (left panel), and a band at 27 kDa which cross-reacts with anti-Rab5 antibodies (right panel).

**Figure 3**




Immunodepletion of GDI and Rab5 from cytosolic extracts. (a) Western blot analysis of bovine brain cytosol immunodepleted of GDI using affinity-purified polyclonal sheep antibodies specific for GDI (see Materials and methods). Pellets (P) and supernatants (S) after immunodepletion with non-specific sheep antiserum (N/S), anti-peptide 1 (αGDI1) and anti-peptide 2 (αGDI2) were immunoblotted using anti-GDI antiserum. (b) Effect of immunodepletion of GDI on adaptor-dependent stimulation of sequestration. Cytosol was treated with either non-specific serum (N/S), anti-peptide 1 (αGDI1) or anti-peptide 2 (αGDI2), coupled to protein-G–sepharose and then assayed for the ability to support adaptor-dependent transferrin sequestration. The fractions used correspond to the supernatants shown in Figure 3a. Purified Rab5–GDI (1 μg) was added to anti-peptide 1-depleted cytosol as indicated. (c) Western blot analysis of bovine brain cytosol immunodepleted of Rab5 using anti-Rab5 antiserum coupled to protein-A–sepharose. Pellets (P) and supernatants (S) after immunodepletion with non-specific rabbit serum (N/S) or anti-Rab5 antiserum (αRab5). (d) Effect of immunodepletion of Rab5 on adaptor-dependent transferrin sequestration. Cytosol was treated with either non-specific serum (N/S) or anti-Rab5 antibodies (αRab5) coupled to protein-A–Sepharose and then assayed for the ability to support adaptor-dependent transferrin sequestration. The fractions used correspond to the supernatants shown in Figure 3c. Purified Rab5–GDI (1 μg) was added to Rab5-depleted cytosol as indicated. (e) Western blot of the cytosolic fraction after phosphocellulose chromatography using antibodies raised against Rab1, Rab4, Rab5 and Rab6. (f) Western blots using antibodies against Rab1, Rab4, Rab5 and Rab6 showing the fractions in which each of these proteins was most enriched during the purification of Rab5–GDI. RRFT, flow-through from reactive red column; QFT, flow-through from MonoQ; PCFT, flow-through from phosphocellulose column. Adaptor-dependent transferrin sequestration of each of these fractions was measured (activity).

cytosol. Antiserum against Rab5 almost quantitatively depleted Rab5 from cytosol (Figure 3c). Addition of depleted cytosol to permeabilised cells led to > 90% reduction in adaptor-dependent transferrin sequestration (Figure 3d), demonstrating that Rab5–GDI represents the critical component in cytosol that is essential for adaptor-dependent transferrin sequestration. Purified Rab5–GDI rescued the ability of cytosol treated with both anti-GDI and anti-Rab5 antibodies to support adaptor-dependent transferrin sequestration (Figure 3b,d). Depletion of Rab1 from cytosol had no effect on the ability of cytosol to support adaptor-dependent sequestration (data not shown). The active fraction obtained after chromatography on phosphocellulose was immunoblotted for Rab1, Rab4, Rab5 and Rab6. Only Rab5 was detected in this purified fraction (Figure 3e). Furthermore, during the course of the purification, we identified, by western blotting, fractions which are highly enriched in other Rab–GDI complexes, for example, containing Rab1, Rab4 and Rab6 (Figure 3f). None of these fractions could support adaptor-dependent transferrin sequestration, however (Figure 3f).

The association of Rab proteins with their target membranes has been shown to be coupled to nucleotide exchange [25,26]. We next addressed whether or not Rab5–GDI involvement in ligand sequestration into invaginated coated pits also required nucleotide exchange. We used a recombinant mutant form of Rab5 which preferentially binds xanthosine 5′-triphosphate (XTP), REP-1:his-Rab5$^{D136N}$. In the presence of XTP, this mutant protein supports endosome–endosome fusion to the same extent as recombinant wild-type Rab5 [29]. Similarly, REP-1:his-Rab5$^{D136N}$ was capable of supporting adaptor-dependent transferrin sequestration to the same extent, and within the same concentration range, as biochemically purified Rab5–GDI. Importantly, this effect was completely dependent on the presence of XTP, demonstrating that nucleotide exchange on Rab5 is essential for adaptor-dependent ligand sequestration (Figure 4).

### GDI inhibits coated pit assembly and scission

A number of studies have demonstrated that GDI acts as an inhibitor of transport reactions *in vitro* by solubilising

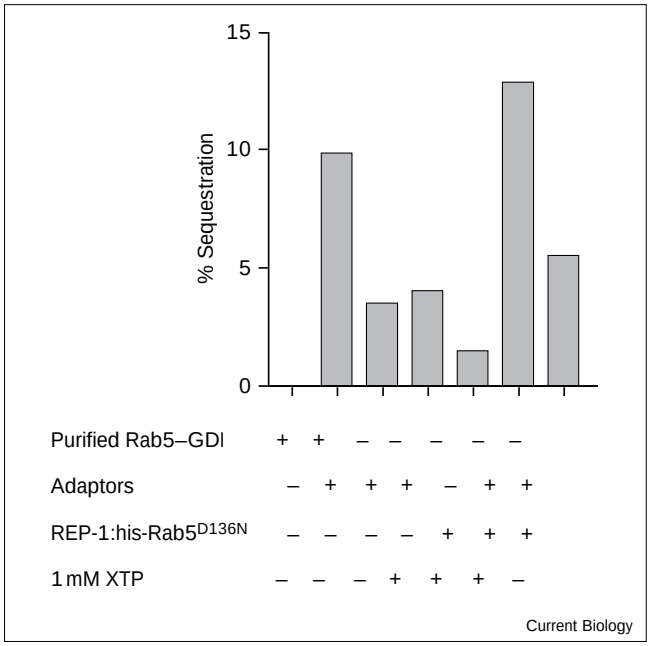
Rab proteins from the membrane (see references within [24]). Given that, in our permeabilised cell system, exogenously added adaptors stimulate only a limited number of steps on the pathway to coated vesicle formation, we wanted to investigate whether Rab5 was required at other stages in coated vesicle formation. Permeabilised cells were incubated with increasing amounts of GDI purified from bovine brain [30], and the extent of transferrin sequestration into deeply invaginated coated pits and its internalisation into coated vesicles was measured using the assays for avidin inaccessibility, MesNa resistance and adaptor-dependent ligand sequestration. Figure 5a–c shows that in all three assays GDI was inhibitory, with half-maximal inhibition occurring at ~0.6 μM and full inhibition occurring at 2 μM in each assay. This inhibitory effect correlated with the removal of 47% of Rab5 from the membranes as determined by western blotting (Figure 5d). These data demonstrate that Rab5 is required at all stages in coated vesicle formation.

### Rab5–GDI acts after coat protein recruitment to promote sequestration

Rab5–GDI supports adaptor-dependent ligand sequestration and adaptors have previously been shown to act at an early stage(s) of coat assembly [12]. Rab5–GDI could, therefore, act either to initiate new coated pits or to promote sequestration. To further characterise the stage at which Rab5–GDI acts, we carried out a morphological analysis of the role of the complex and adaptors in this system. Permeabilised cells were incubated under a variety of conditions and processed for electron microscopy. A quantitative analysis of the number of coated pits per nucleus was carried out using the disector method, which samples particles with equal probability regardless of their size and provides an unbiased estimate of the number of particles [10,31]. Rab5–GDI alone had no effect on the numbers of coated pits at the plasma membrane (Table 2). In contrast, adaptors alone, in the presence of ATP, led to a striking 2–3-fold increase in the number of coated pits per nucleus (Table 2). The increase was absolutely dependent on the presence of ATP: in the presence of an ATP-depleting system, adaptors were not capable of effecting *de novo* coated pit formation.

Our biochemical assays have demonstrated that the extent of sequestration of transferrin seen in the presence of adaptors and ATP alone is modest. Maximum sequestration requires the presence of cytosol and, as described above, this cytosolic requirement can be completely fulfilled by Rab5–GDI. The Rab5–GDI complex itself does not enhance sequestration in the absence of adaptors. Thus, although coat proteins in the presence of ATP are capable of forming new coated pits, these pits can only sequester ligand in the presence of Rab5–GDI, demonstrating that Rab5–GDI acts after the recruitment of coat proteins.

**Figure 4**

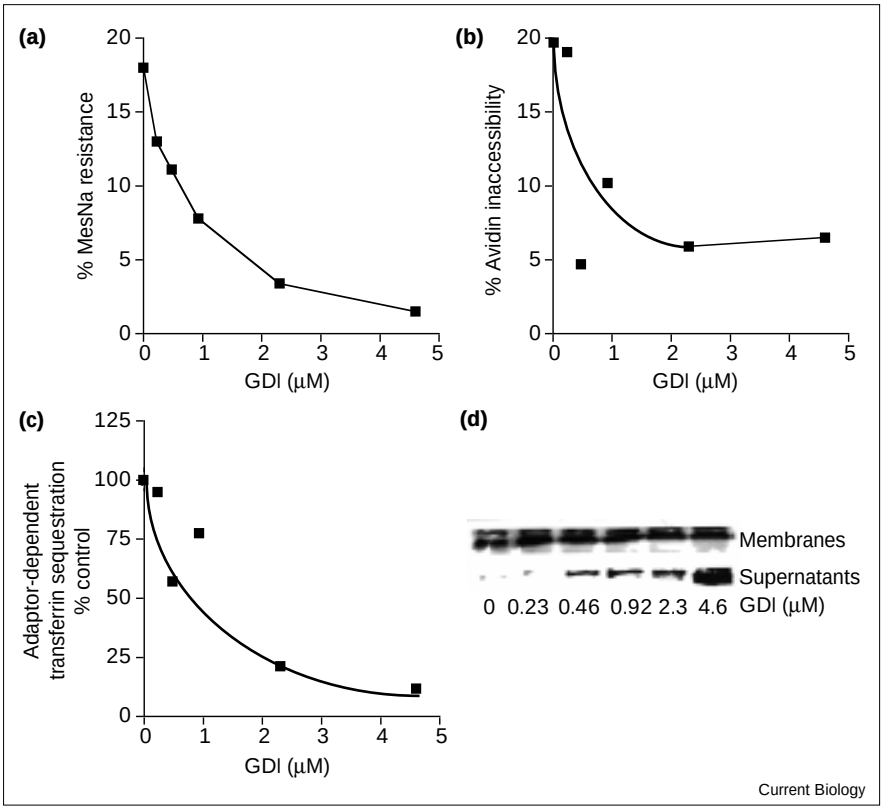



REP-1:his-Rab5$^{D136N}$ complex can support adaptor-dependent transferrin sequestration in the presence of XTP. The extent of transferrin sequestration in permeabilised cells was measured in the presence of purified Rab5–GDI (50 nM), REP-1:his-Rab5$^{D136N}$ (50 nM), adaptors (10 μg), and XTP (1 mM) as indicated. Background sequestration measured in the presence of an energy-regenerating system alone has been subtracted, and each value represents the mean of duplicate samples which differed by less than 10% of each value.

### A role for clathrin in adaptor-dependent ligand sequestration

In the presence of Rab5–GDI and purified adaptors, transferrin was sequestered into coated pits, raising the question as to the source of clathrin. It should be noted that, although > 95% of the soluble material, as assayed by release of lactic dehydrogenase, is lost from the cells during the permeabilisation process [10], the membranes are not rigorously stripped of peripheral membrane proteins. In order to investigate whether clathrin might be recruited from the A431 cell membranes for incorporation into coated pits, the permeabilised cell membranes were stripped of clathrin using 100 mM Tris pH 7.8. This process resulted in the loss of ~90% of the membrane-associated clathrin (Figure 6a). When these stripped membranes were assayed for adaptor-dependent transferrin sequestration, they retained the ability to sequester transferrin in the presence of cytosol (Figure 6b). This finding was consistent with our previous results showing that clathrin may be recruited from the cytosol to participate in coated pit assembly [12]. No sequestration was observed in the presence of purified Rab5–GDI and adaptors, however (Figure 6b). This contrasts with unstripped cells, in which addition of adaptors and Rab5–GDI is sufficient

**Figure 5**




GDI inhibits all of the stage-specific assays for clathrin-coated vesicle formation. GDI purified from bovine brain was added to permeabilised cells at the indicated concentrations, (**a**,**b**) in the presence of cytosol (1.5 mg/ml), ATP and BSST, and (**c**) in the presence of reactive red fraction (0.09 mg/ml), ATP, BSST and purified adaptors (10 μg). The final volume of the reaction mix was 40 μl. (a) Coated vesicle budding was measured using the MesNa resistance assay. (b) Coated pit assembly, invagination and scission was measured using the avidin inaccessibility assay. (c) The invagination of new coated pits was measured using the adaptor-dependent assay. The results shown are from a typical experiment performed three times and each point represents the mean of duplicate values which differed from each other by less than 10%. (**d**) Membranes were incubated with GDI at the indicated concentrations for 30 min at 37°C. The membranes were then pelleted and both membrane pellets and supernatants were subjected to SDS–PAGE followed by western blotting using anti-Rab5 antibodies.

to stimulate coated pit assembly in permeabilised cells (Figure 1b). The ability to sequester transferrin was restored in the stripped cells in the presence of purified clathrin. As there is no clathrin in our purified preparations of Rab5–GDI (data not shown), it seems likely that clathrin is recruited from the membranes of unstripped, permeabilised cells to participate in transferrin sequestration.

**Table 2**

**Effect of adaptors, Rab5–GDI and ATP on coated pit assembly in permeabilised A431 cells.**

| Incubation conditions | Number of coated pits/nucleus ± coefficient of error |
|---|---|
| +ATP +low cytosol | 661 ± 59 |
| +ATP +low cytosol, +adaptors | 933 ± 81 |
| +ATP +Rab5–GDI | 494 ± 43 |
| +ATP +Rab5–GDI +adaptors | 1094 ± 105 |
| +ATP +adaptors | 1354 ± 96 |
| –ATP +Rab5–GDI +adaptors | 416 ± 39 |

Numbers of coated pits per nucleus were counted using the double disector method [31]. The decrease in the number of coated pits per nucleus in the presence of adaptors and Rab5–GDI or cytosol does not appear to be significant because in two other experiments where the number of coated pits per nucleus were counted in the presence of adaptors and ATP only, the numbers obtained were 921 ± 62 and 1148 ± 117.

## Discussion

Many questions remain unanswered concerning the formation of clathrin-coated pits and coated vesicles [9] and to address these questions it is necessary to identify all of the molecular components required for these processes. The reconstitution of clathrin-coated pit assembly and coated vesicle formation in permeabilised A431 cells has demonstrated that cytosol and ATP promote both new coated pit formation and coated vesicle budding [10–12]. Because adaptors are limiting in cytosol, addition of purified adaptors stimulates coated pit assembly as measured by ligand sequestration into deeply invaginated pits, and this stimulation requires both clathrin and other cytosolic components [12]. In this study, we have purified the cytosolic component(s) required for adaptor-dependent transferrin sequestration and shown it to be a complex of Rab5 and GDI. We also show that the Rab5–GDI complex acts after new coated pits have assembled and is essential for ligand sequestration. Thus, the components that will subsequently be required for correct targeting and fusion are built into the nascent transport vesicle and indeed are necessary for clathrin-coated pits to sequester ligand.

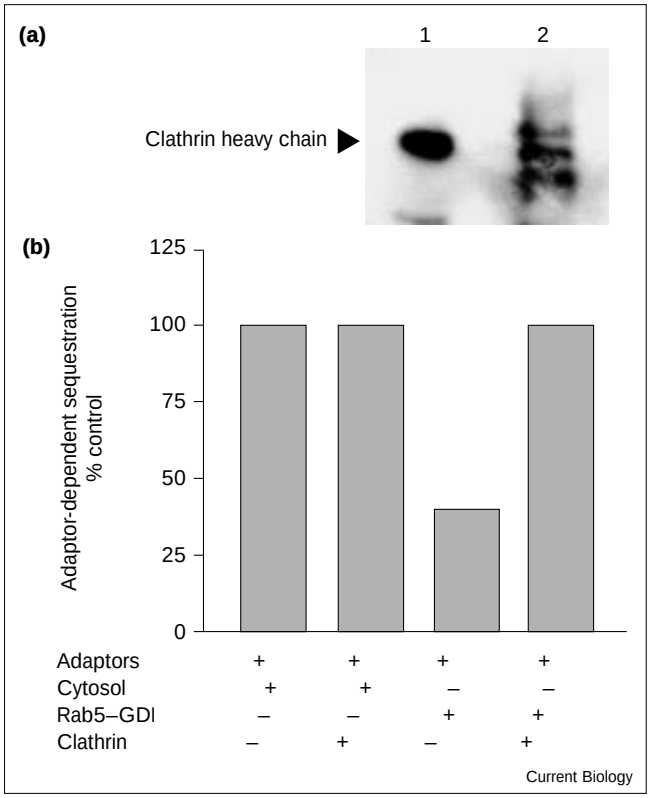
### Rab5–GDI is essential for clathrin-coated pit function

In this study, we have shown that new coated pits form in permeabilised A431 cells in the presence of added

adaptors and an energy source. Efficient sequestration of ligand requires the presence of cytosolic factors, however. Making no assumptions as to the outcome, we have fractionated bovine brain cytosol and have identified Rab5–GDI as the essential cytosolic component required for adaptor-dependent transferrin sequestration. We believe that Rab5–GDI and not other Rab–GDI complexes represents the cytosolic activity required for adaptor-dependent transferrin sequestration for the following reasons. Firstly, immunodepletion of Rab5 from cytosol abolishes almost all of the activity required for adaptor-dependent transferrin sequestration, indicating that other cytosolic Rab protein complexes cannot substitute for Rab5–GDI. Secondly, recombinant REP-1:his-Rab5$^{D136N}$ [29] can also support adaptor-dependent sequestration with the same concentration optimum (50 nM) as the purified Rab5–GDI complex. Furthermore, in the course of the purification we identified fractions that were highly enriched in other Rab–GDI complexes, containing for example Rab1, Rab4 and Rab6. None of these fractions could support adaptor-dependent transferrin sequestration, however. We believe that Rab5–GDI completely fulfils the requirement for cytosol in adaptor-dependent transferrin sequestration in the permeabilised A431 cell system because the extent of adaptor-dependent sequestration is the same for cytosol as it is for both the biochemically purified complex and the recombinant REP-1:his-Rab5$^{D136N}$ complex.

Adaptor-dependent transferrin sequestration never exceeds 10–15% of the total transferrin being sequestered. This is as expected because this step measures the sequestration of transferrin receptors into newly formed coated pits, and there are only a finite number of receptors that can be included in these pits. The concentration of Rab5–GDI that supports adaptor-dependent sequestration is similar to that required to promote endosome–endosome fusion *in vitro* [22,29] and, as expected, is less than the total cellular concentration of GDI, which has been estimated in CHO cells as ~170 nM, with higher levels in brain and liver (see references in [24]). Nucleotide exchange is required for the stimulatory effects of Rab5 on early endosome fusion [29] and also for the ypt1-dependent fusion of ER-derived vesicles [32]. Similarly, Rab5–GDI-driven ligand sequestration into deeply invaginated pits is coupled to nucleotide exchange. The ability of the mutant REP-1:his-Rab5$^{D136N}$ complex to support ligand sequestration is dependent on the presence of XTP. Given that nucleotide exchange is required for ligand sequestration and that GDI inhibits this reaction (see below), it seems likely that it is the recruitment of Rab5 to the membrane which is critical for ligand sequestration. Presumably GDI is acting to effect the delivery of Rab5 to the membrane, as has been demonstrated in other systems [25,26].

**Figure 6**




Permeabilised cells stripped of clathrin can use membrane clathrin to support adaptor-dependent transferrin sequestration. (a) Western blot analysis of clathrin associated with permeabilised cells. Clathrin associated with membranes from $10^6$ A431 cells permeabilised in KSHM (lane 1) or after stripping with 100 mM Tris, pH 7.8 (lane 2). (b) Permeabilised cell membranes were stripped following permeabilisation by incubation in 100 mM Tris pH 7.8 for 10 min at 0°C. Membranes were then pelleted and resuspended in KSHM. These membranes were then assayed for adaptor-dependent ligand sequestration in the presence of cytosol (30 μg), Rab5–GDI (0.4 μg) and/or clathrin (10 μg) as indicated. Data are from a typical experiment and each point represents the mean of duplicate samples which differed by less than 10%.

It should be noted that although we have identified Rab5–GDI as the cytosolic component required for adaptor-dependent transferrin sequestration in permeabilised A431 cells, we would expect that additional factors essential for the formation of clathrin-coated pits and vesicles would have been previously recruited to the permeabilised cell membranes. Possible candidate proteins might include a member of the ADP-ribosylation factor (ARF) family of small GTPases, shown to be involved in the formation of other coated vesicles within the cell [33,34], or non-neuronal homologues of proteins that may be involved in the clathrin-mediated uptake of membrane at the synapse [35,36]. In our study, it appears likely that clathrin is recruited from the permeabilised cell membranes to participate in new coated pit assembly, and

similarly other components may remain associated with the A431 cell membranes following permeabilisation.

Further evidence for the direct involvement of Rab5 in clathrin-coated pit assembly and coated vesicle formation comes from our studies of the effect of GDI on these processes. GDI not only markedly inhibits adaptor-dependent sequestration of transferrin but, interestingly, also inhibits all other steps on the pathway to coated vesicle formation. Exogenous adaptors and Rab5–GDI have no effect on the later stages of coated vesicle formation, as measured by the MesNa assay ([12,37]; H.M. and E.S., unpublished observations). Previous studies have demonstrated that the formation of clathrin-coated vesicles in permeabilised A431 cells appears to result from a population of coated pits 'primed' for scission upon permeabilisation [11]. These pits presumably have already recruited many essential components, including Rab5. Although the exogenously added Rab5–GDI did not support the scission step in permeabilised A431 cells, its removal inhibited coated vesicle formation. These data are consistent with a role for Rab5–GDI in all stages of clathrin-coated vesicle formation.

A number of lines of evidence demonstrate that Rab5 plays a major role in the fusion of uncoated clathrin-coated vesicles and early endosomes; indeed Rab5 has been shown to be a component of clathrin-coated vesicles [23]. Two possibilities could explain how Rab5 might be incorporated into coated vesicles: it could be recruited as part of the growing pit, or it could be recruited to clathrin-coated vesicles as has been shown *in vitro* [38]. The results of our study demonstrate that Rab5 is recruited during the process of clathrin-coated pit formation. In addition, recruitment at this early stage is critical for coat invagination and coated vesicle scission. These studies provide the first direct evidence that Rab5–GDI is required for clathrin-coated vesicle formation, and they may explain earlier data demonstrating that the initial rates of transferrin internalisation were enhanced in cells overexpressing wild-type Rab5 [23]. Rab5 is clearly limiting in the endocytic pathway, and our evidence that it is directly involved in clathrin-coated pit formation is consistent with the finding that Rab5 overexpression stimulates transferrin uptake. Similarly, the overexpression studies demonstrate that the life-time of a coated pit is reduced by 50% in cells transfected with wild-type Rab5 [23]. In intact cells, other components necessary for coated pit budding may not be limiting and hence, in the presence of increased levels of Rab5, the rate of budding from the cell surface will be enhanced.

#### Coat proteins and ATP are sufficient for *de novo* coated pit assembly

We have examined the role of individual purified components in coated pit assembly in permeabilised cells using quantitative electron microscopy. Addition of adaptors resulted in at least a two-fold increase in the number of coated pits at the cell surface, but only in the presence of ATP. In permeabilised cells, exogenously added adaptors and ATP were sufficient for the assembly of new coated pits, which also showed the distinctive bristle-like coat that is characteristic of clathrin-coated pits. It seems probable that there is substantial recruitment of clathrin from the permeabilised cell membranes. This idea is supported by the requirement for clathrin for efficient sequestration of ligand in permeabilised cells that have been subjected to a more stringent washing procedure. We cannot rule out the possibility, however, that residual cytosolic clathrin that has not been removed from the cells during the permeabilisation procedure is sufficient to support the formation of new coated pits in this system.

Depletion of ATP prevents *de novo* coated pit assembly. This result extends previous studies showing that new coated pit formation is dependent on the presence of both cytosol and ATP [10]. Similarly, in permeabilised NRK cells, the recruitment of exogenously added adaptors to the plasma membrane has been shown to be ATP-dependent [39]. It will be interesting to define the precise role played by ATP in new pit formation: ATP could be essential for new pit assembly, or alternatively it could be required for the release of clathrin from the permeabilised cell membranes.

#### Rab5–GDI acts after coat assembly

By a combination of morphological and biochemical assays, we have established that Rab5–GDI acts after coat assembly and is essential for the efficient sequestration of ligand. Figure 7 shows a schematic model of how we believe Rab5–GDI acts in this system. Coat proteins are recruited to the membrane in an ATP-dependent manner. Although lattices are formed under these conditions, these lattices become capable of ligand sequestration only after recruitment of Rab5–GDI. Although exogenous Rab5 does not support sequestration or internalisation in the absence of adaptors, removal of Rab5 inhibits these processes. Our conclusion on the basis of these data is that Rab5 is incorporated early in clathrin-coated pit assembly and hence is required for the sequestration of ligand into newly formed coated pits (measured by the adaptor-dependent signal). The pre-existing pits that sequester or internalise ligand have therefore already recruited Rab5, so exogenously added Rab5 has no effect. As expected, immunodepletion of Rab5 from cytosol has no effect on the ability of the cytosol to support sequestration or internalisation through pre-existing pits in the avidin and MesNa assays (data not shown).

Our data support the hypothesis that the processes of coat assembly and invagination are separately regulated, consistent with observations made in this and other systems. It has been previously shown that A431 cell membranes

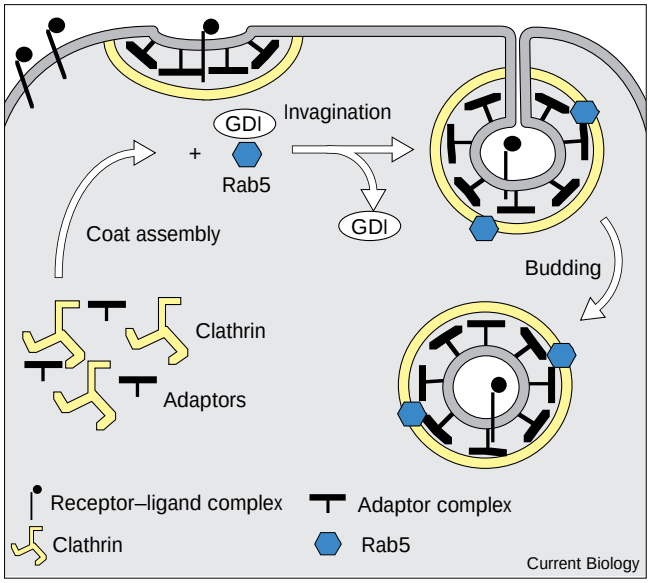
could be 'primed' by pre-incubation with adaptors. The unbound adaptors could then be removed and sequestration could still occur, provided that cytosol and ATP were added in the subsequent reaction [12]. Purified Rab5–GDI is also able to support sequestration by adaptor-primed membranes (H.M. and E.S., unpublished observations). Other studies show that high levels of transferrin receptor expression can promote the formation of clathrin lattices with up to 30% of the cell surface being covered. There is no increase in the number of invaginated coated pits, however [40]. These lattices appear to be relatively long-lived, suggesting that components required for invagination are limiting in this system.

#### Implications for vesicular membrane traffic

The requirement for Rab5–GDI for sequestration indicates that the recruitment of essential components of the targeting and fusion machinery necessary for later transport steps is closely coupled to the formation of functional clathrin-coated vesicles. Although this is the first demonstration of a direct role for a Rab protein in clathrin-coated pit formation, other studies have implicated a role for functional Rab1 in the budding of COP I-coated vesicles from the ER [41–43]. Rab1 is also required at later stages of docking and fusion [44]. Yeast transport vesicles can recruit ypt1, the yeast homologue of Rab1, after budding has occurred [45,46]. Similarly, clathrin-coated vesicles from mammalian cells can recruit Rab5 [38], although it has also been reported that during the purification of coated vesicles the associated Rab5 is lost [23], and this Rab5 might then be able to rebind the vesicles. Our demonstration of a direct role for Rab5–GDI in clathrin-coated pit formation suggests that there are strong mechanistic similarities in the way that different transport vesicles are formed.

We have shown that Rab5–GDI is required for clathrin-coated pit invagination and it is intriguing to speculate about the mechanism of action of the complex in this process. One possibility is that Rab5–GDI has a direct role in promoting sequestration, either by driving invagination or by receptor recruitment. A second possibility is that there is a molecular 'sensor' that ensures that Rab5 is recruited to the growing pit for its subsequent role in targeting and fusion reactions, in other words, there is a quality-control mechanism which ensures that functional transport vesicles only form if they contain all of the components needed for the next stage in transport. A third possibility is that the role of Rab5 in targeting and fusion of endocytic vesicles is mediated by events that occur early in coated pit formation. In this context, it is interesting to note that recent studies from other systems have implicated NSF (N-ethylmaleimide-sensitive fusion protein) and SNAPs (soluble NSF attachment proteins), components known to be involved in vesicle docking and fusion, in pre-docking or vesicle budding events [19,47].

**Figure 7**




Proposed role of Rab5–GDI in clathrin-coated pit formation. This figure summarises the proposed role of Rab5–GDI in clathrin-coated pit formation, on the basis of our studies. Coat proteins are recruited to the membrane of permeabilised cells in an ATP-dependent fashion. GDI delivers Rab5 to the membrane to promote efficient sequestration of ligand. Nucleotide exchange occurs and GDI dissociates. Rab5 is needed for all steps on the pathway to coated vesicle formation.

Given that these components are known to interact with SNAREs, a number of hypotheses have been proposed to account for the action of these components, including reactivation of SNAREs following a fusion event, and regulation of the interaction of SNAREs with fusion partners or with other essential components of the transport machinery [19]. The Rab/ypt1 family has been proposed to have related roles in the regulation of SNAREs, so it may well be that Rab proteins also perform part or all of their function at a very early stage of vesicle budding.

In conclusion, we provide a model whereby the invagination of a coated pit and its ability to sequester ligand is dependent on the recruitment of Rab5 from the cytosol, where it exists as a complex with GDI. An investigation of the events upstream and downstream of the recruitment of Rab5–GDI should further elucidate the mechanism of coated pit assembly and function.

## Materials and methods

### *Preparation of Rab5–GDI*

Cytosol was prepared from bovine brains (PelFreez) stored at –80°C. Thawed brains were homogenised in an equal volume of KSHM buffer (100 mM potassium acetate, 85 mM sucrose, 1 mM $MgCl_2$, 20 mM HEPES pH 7.4). After centrifugation at 10,000 rpm for 30 min in a Beckman JA10 rotor, the supernatant was further centrifuged at 25,000 rpm in a Beckman Ti45 rotor. The supernatant was applied to a 300 ml reactive red dye matrix (Sigma) equilibrated in KSHM buffer.

After washing, Rab5–GDI was eluted from the column with 7.5 mM pyruvic acid in KSHM and concentrated by 60% ammonium sulphate precipitation. The protein precipitates were resuspended in KSHM. Following dialysis, the protein was loaded onto a MonoQ ion-exchange column (Pharmacia) equilibrated in KSHM. After washing, Rab5–GDI was eluted using a linear gradient between 0.1 and 0.28 M KCl in KSHM. Fractions containing Rab5–GDI were pooled and dialysed overnight against 0.02 M sodium acetate pH 5.2, containing 0.5 M sucrose. This sample was then applied to a 1 ml phosphocellulose column, prepared according to manufacturers' instructions and equilibrated in start buffer (0.02 M sodium acetate pH 5.2, 0.5 M sucrose). Rab5–GDI flowed through the column, whereas almost all of the remaining contaminating proteins in the preparation bound to the phosphocellulose. The flow-through was concentrated (0.1–0.2 mg/ml). Gel filtration of this fraction was carried out on a Pharmacia SMART system using a Superdex 200 column equilibrated in KSHM containing 100 μM GDP. The final purified fraction consisted of two major bands of 58 kDa and 27 kDa. In some preparations, there was also a minor contaminant of 23 kDa (also present in some inactive fractions). Sequence analysis of this band has shown it to be thiol-specific antioxidant protein (data not shown). Purified fractions were stored at –80°C.

### *Antibodies*

Antiserum against GDI was raised in sheep by the Scottish Antibody Production Unit. Peptides corresponding to the following sequences: (1) KRKQNDVFGEADQ, and (2) GESSSITPLEELYK (in single-letter amino-acid code), were coupled to BSA and keyhole limpet haemocyanin using glutaraldehyde.

### *Assay for adaptor-dependent stimulation of sequestration*

The assays for avidin inaccessibility, MesNa resistance and adaptor-dependent stimulation of transferrin sequestration were carried out as previously described [48,49].

### *Other methods*

All other methods were carried out according to published procedures. Further details are available as supplementary material.

### *Supplementary material*

Additional methodological detail and two figures showing Superdex 200 fraction profiles, and electron micrographs used to perform the quantitation shown in Table 2, are available with the internet version of this paper.

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